Detection of Human Immunoglobulins G and M Antibodies to Rift Valley Fever Virus by Enzyme-Linked Immunosorbent Assay

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Rift Valley fever virus (RVFV) is an important human and animal pathogen in Africa and has been responsible for infections in travelers. Because of the aerosol infectivity and risk of dissemination of the virus, a need exists for simple, safe, serological tests for diagnosis. An enzyme-linked immunosorbent assay (ELISA) was developed to detect RVFV-specific immunoglobulins (immunoglobulin G [IgG] and IgM). In the test, a betapropiolactone-inactivated, sucrose-acetone-extracted, suckling mouse liver RVFV antigen was captured by mouse RVFV antibodies adsorbed to polystyrene plates. The test sample (human serum) was then added, and the binding of specific antibodies was indicated by alkaline phosphatase-conjugated swine anti-human IgG or IgM. A μ-capture IgM ELISA was also developed by using polystyrene plates coated with goat anti-human IgM incubated successively with serum sample, RVFV antigen, and indicator antibodies. The ELISA for RVFV-specific IgG proved to be more sensitive than hemagglutination inhibition or complement fixation tests and almost as sensitive as the plaque reduction neutralization test in detecting specific antibodies in human sera after vaccination. The two ELISA IgM tests could detect specific IgM antibodies during the first 6 weeks after RVFV vaccination. Three injections of inactivated vaccine were given on days 0, 6 to 8, and 32 to 34. ELISA IgM values for sera obtained on days 6 to 8 were negative or in the lower range of significance, on days 32 to 34 they were strongly positive, and on days 42 to 52 they were waning. Later sera were negative. The plaque reduction neutralization test was negative on days 6 to 8 but rose progressively in later samples. These findings suggest that the three doses of RVFV vaccine induce a prolonged primary antibody response. The ELISA IgM could become an important tool for early diagnosis in acute human infection. A number of African sera, some of which were positive for RVFV by plaque reduction neutralization test, were also tested by ELISA IgG. There was good agreement between both tests.

Rift Valley fever (RVF) is an economically important arthropod-borne virus disease in Africa, primarily affecting sheep, goats, and cattle (13). Until 1975, RVF was considered to be a benign febrile illness in humans; however, that year, fatal human hemorrhagic fever and encephalitis were seen in association with RVF in South Africa (23) and Zimbabwe (20). During RVF epidemics in Egypt in 1977 and 1978, a large number of human fatalities were reported (11).

The clinical diagnosis of an RVF epizootic in an area in which the virus is known to occur may be relatively easy because most pregnant ewes and cows abort owing to the infection, and a high mortality is often seen among newborn lambs (13). Human disease may occur simultaneously, particularly in exposed veterinarians and slaughterhouse workers. However, diagnosis is often delayed when RVF extends into new regions, and laboratory confirmation is necessary even in the presence of suspicious disease activity (17).

Methods for measurement of antibodies to RVF virus (RVFV) have been developed. The hemagglutination inhibition (HI), complement fixation (CF), indirect immunofluorescence, and neutralization tests can be used to detect antibodies after the natural infection.

Effective RVF vaccines are available for both animal and human use (3, 7, 14). It is important to measure the antibody response after immunization, and the plaque reduction neutralization test (PRNT) is often used since it is a specific (21) and sensitive test which is able to detect even low amounts

of antibodies and since neutralizing antibodies are thought to correlate with protection (7).

The enzyme-linked immunosorbent assay (ELISA) has been used for the detection of antibodies in other viral diseases and found to be a rapid, sensitive, and specific method (6, 8, 24). The ELISA system was therefore applied for the detection of RVF antibodies of the immunoglobulin G (IgG) and IgM classes. This study evaluates the method for detection of RVF antibodies and compares results by ELISA, HI, CF, and PRNT tests with sera collected during an RVF vaccination program. Both ELISA systems (IgM and IgG) were also evaluated by using African sera collected during seroepidemiological surveys in Sierra Leone, the Central African Republic, and the Sinai.

MATERIALS AND METHODS

Virus strains. Zagazig Hospital (ZH) 501 strain of RVFV was originally isolated by James Meegan, NAMRU-3, Cairo, Egypt, from a fatal human case of hemorrhagic fever in Egypt in 1977, and it was used in the second fetal rhesus lung cell culture passage. The Entebbe strain of RVFV was isolated in Uganda from a mosquito in 1944 and passaged intraperitoneally 184 times in mice (14).

Antigen. A betapropiolactone-inactivated, sucrose-acetone-extracted, mouse liver antigen was used for ELISA. The antigen had been manufactured by Government Services Division, Salk Institute, Swiftwater, Pa., for RVF HI serology, using the Entebbe strain of RVFV (22).

Vaccine. The RVF vaccine used for immunization was

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manufactured for the U.S. Army Medical Research and Development Command by the National Drug Co., Swiftwater, Pa. The vaccine was prepared from the Entebbe strain of RVFV propagated on primary African green monkey kidney cell cultures and Formalin-inactivated.

Immune reagents. Antibodies against RVFV were obtained from rabbit sera and mouse ascitic fluids. Rabbits were immunized with a single intravenous injection of mouse serum containing the Entebbe strain of RVFV. Mice received 0.1 ml of 10% suckling mouse liver homogenate of the ZH 501 strain intraperitoneally and were treated 24 h later with 3 mg of polyriboinosinic-polyribocytidylic acid per kg complexed with poly-L-lysine and carboxymethyl cellulose subcutaneously to prevent lethal liver disease (7). Subsequently, they received four to six intraperitoneal injections of the homogenate emulsified in complete Freund adjuvant before ascites were induced with sarcoma 180.

Swine anti-human IgG, swine anti-rabbit IgG, and swine anti-human IgG conjugated with alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) and goat μ-chain specific anti-human IgM serum (Cappel Laboratories, Downingtown, Pa.) were obtained commercially. Optimal dilutions of all reagents used in the ELISA for IgG or IgM detection were determined for each batch by chessboard titration.

Human sera. A total of 76 sera from 21 Swedish male United Nations soldiers immunized with the RVF vaccine (receiving three injections of 1 ml each on days 0, 6 to 8, and 32 to 34) and bled on days 0, 6 to 8, 32 to 34, and 42 to 52 were tested by ELISA IgG, ELISA IgM, PRNT, and the HI and CF tests. Sera from unimmunized soldiers were used to establish criteria for positivity in tests of vaccinees. One group of 15 individuals was bled 6 months after vaccination and tested by PRNT, ELISA IgG, and ELISA IgM. A total of 90 African sera were collected during an epidemiological investigation of apparently healthy populations and were furnished by J. P. Gonzales, ORSTOM, Institute Pasteur, Bangui, Central African Republic. All African sera were tested by PRNT, ELISA IgG, and ELISA IgM. Nine sera with antibodies to Sandfly fever Sicilian virus by PRNT were also tested by ELISA IgG and ELISA IgM (4). Also, the sera of 57 Bedouins, living in the Sinai peninsula, were tested by PRNT, HI, and ELISA IgG.

Affinity chromatography. Affinity-purified antibodies were used to increase the sensitivity and decrease the background activity of the ELISA test (10). The inactivated RVFV antigen was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by using 1 g of gel and 2 ml of undiluted hemagglutination antigen (2). Rabbit or mouse ascites fluid (1 ml) was passed through the column. After 30 min of incubation at room temperature, the gel was washed with phosphate-buffered saline, and the adsorbed anti-RVF immunoglobulins were eluted from the column with 0.2 M glycine (pH 2.8). After elution, the pH was neutralized by Tris buffer, and the immunoglobulins were than dialyzed against phosphate-buffered saline overnight and concentrated 10 times in a Minicon concentrator. The final protein concentrations calculated from the optical density (OD) at 280 nm were 1.00 and 0.52 mg/ml for the rabbit and mouse antibodies, respectively.

ELISA procedures for detection of RVFV IgG antibodies. A double antibody (sandwich) ELISA was used to measure RVFV IgG antibodies. Affinity-purified mouse RVFV antibodies were diluted 1:160 in coating buffer (0.05 M sodium carbonate, pH 9.5 to 9.7) and added (100 μl) to 60 of the 96 wells (excluding the outer rows) of polystyrene microtiter plates (Cooke M 29 AR; Dynatech Laboratories). After 1 h of

incubation at 37°C, the plates were washed in rinsing solution (saline with 0.05% Tween 20).

The RVFV antigen, diluted 1:100 in ELISA buffer (PBS without Mg and Ca with 0.05% Tween 20 and 0.5% bovine serum albumin), was added to each well (100 μl), incubated for 1 h at 37°C, and then washed four times in rinsing solution. The human test serum was diluted 1:40 in ELISA buffer supplemented with 1% normal mouse serum and added (100 μl) to duplicate wells. All human test sera were added, diluted as above, to duplicate wells treated as described above but without antigen. Plates were incubated at 37°C for 1 h. After being washed four times in rinsing solution, 100 μl of alkaline phosphatase-conjugated swine anti-human IgG, diluted 1:200 in ELISA buffer, was added. After incubation (1 h in 37°C) and four washes in rinsing solution, 100 μl of substrate (p-nitrophenyl-phosphate; Sigma Chemical Co., St. Louis, Mo.) diluted in diethanolamine buffer (1 M diethanolamine [pH 9.8], 0.5 mM $MgCl_2$) was added. The reaction was read after 30 min at room temperature by a spectrophotometer set at 405 nm. A sample was considered positive if the difference in OD between positive (with antigen) and negative (without antigen) control was more than the mean background (tested 30 known negative sera) plus 2 standard deviations.

ELISA procedures for detection of RVFV IgM antibodies. We applied the μ-capture IgM ELISA (6). Goat anti-human IgM (μ-chain-specific) serum was diluted 1:200 in coating buffer and added (100 μl) to 60 of the 96 wells (Cooke M 29 AR plates). After 2 h of incubation at 37°C, the plates were washed four times in rinsing solution. A 100-μl amount of human test serum diluted 1:40 in ELISA buffer was then added and incubated for 1 h at 37°C. After four washes the RVFV hemagglutination antigen diluted 1:100 in ELISA buffer was added and incubated 1 h at 37°C. After being washed four times, 100 μl of the affinity-purified rabbit RVFV antibodies diluted 1:640 in ELISA buffer was added. Incubation (1 h at 37°C) and washing were followed by 100 μl of the alkaline phosphatase-labeled swine anti-rabbit IgG diluted 1:200 in ELISA buffer. After another incubation (1 h at 37°C and washing), 100 μl of substrate p-nitrophenyl-phosphate diluted in diethanolamine buffer was added. The reaction was read after 20 min at room temperature with a spectrophotometer set at 405 nm. As a control all serum samples were tested without the RVFV antigen. A serum sample was considered positive if the difference in OD between test with antigen and test without antigen was more than the mean background (testing 30 known negative sera) plus 2 standard deviations.

For comparison, we also developed an indirect assay for RVFV IgM following the same procedure as that for IgG antibodies, but the human serum diluted 1:40 was incubated for 2 h instead of for 1 h at 37°C. The alkaline phosphatase-labeled swine anti-human IgM serum was diluted 1:75 in ELISA buffer. After adding the substrate the OD was read after 20 min at room temperature.

Problems with false positives because of rheumatoid factor and false negatives because of competing specific IgG have been reported. Serum samples were therefore tested when untreated and absorbed with protein A to remove IgG (1).

Neutralization test. Antibody titers to RVFV were determined by PRNT. Sera were serially diluted fourfold with micropipettes in 96-well microtiter plates. Equal volumes of previously titrated ZH 501 strain of RVFV containing 80 to 100 PFU/25 μl were added to each well. After incubation at 37°C for 60 min, 50 μl of the mixture was inoculated into

each of 2 wells of the tissue culture plate (16 mm) containing 2- to 4-day-old confluent Vero cell monolayers. After 60 min of adsorption, 0.5 ml of agar overlay per well was added (45°C mixture of 1 part 1% agarose and 1 part 2× basal Eagle medium with Earle salts, 17 mM HEPES [N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid], 8% heated calf serum, and 100 U of penicillin and 100 μg of streptomycin per ml). After incubation at 37°C in a 5% CO_2 atmosphere for 3 days, a second overlay was applied that was identical to the first but contained neutral red stain (1:9,000). Plaques were enumerated the next day. An 80% reduction of plaques was used as the index for virus neutralization titers. The lowest serum dilution tested was 1:10. All dilutions were performed in Hanks balanced salt solution buffered to pH 7.2 with 10 mM HEPES and containing 2% heated calf serum and 50 U of penicillin and 50 μg of streptomycin per ml.

CF and HI tests. Antigens for the tests were prepared from livers of RVFV-infected suckling mice by the sucrose-acetone extraction method described by Clarke and Casals (5). The HI method was also performed as described by Clarke and Casals, using acetone extraction to remove nonspecific inhibitors (5). The CF test was performed as described by Shope and Sather (18). The lowest serum dilution was 1:4 for the CF test and 1:10 for the HI test.

RESULTS

ELISA for specific IgG compared with other methods. A total of 76 sera from 21 individuals immunized with RVF vaccine were tested by PRNT and ELISA IgG. Of the 76 sera, 70 were also tested by the HI and CF tests. Sera from day 0 ($n = 21$) and days 6 to 8 ($n = 23$) were found to be negative by all methods (ELISA IgG, PRNT, HI, and CF). When bled on days 32 to 34 all had developed antibodies by PRNT. Figure 1 shows all sera with PRNT titers of 1:10 or greater ($n = 32$) and their corresponding ELISA IgG results. The ELISA IgG was almost as sensitive as the PRNT, missing only one positive serum with a PRNT titer of 1:80. Of the 32 sera with PRNT titers of ≥1:10, 26 were also tested by the HI and CF tests. HI was less sensitive than PRNT, detecting only 4 positive sera out of 11 sera with PRNT titers of 1:160. When tested by CF, all 26 sera were found to be

FIG. 2. Comparison of ELISA and PRNT in late postvaccination and postinfection sera. ELISA IgG OD reading compared with PRNT titer. Sera collected 6 months post-RVF vaccination (X). Sera collected during an epidemiological survey in the Central African Republic containing RVF antibodies (O). Postvaccination sera: slope 0.33, standard error of the slope 0.16, $P < 0.05$. African sera: slope 0.79, standard error of the slope 0.19, $P < 0.01$. Postvaccination and African sera: slope 0.71, standard error of the slope 0.10, $P < 0.01$. The border line between positive and negative was estimated for vaccination sera (lower line) and African sera (upper line) separately.

negative, except one which had a CF titer of 4 and a PRNT titer of 1:640. All sera with negative PRNT results were negative in other tests.

Fifteen sera obtained at 6 months postvaccination were tested by ELISA IgG and PRNT. With the exception of 2 sera (PRNT titers of 1:10 and 1:20), all 15 sera that neutralized RVFV were positive by ELISA IgG. Of 26 PRNT-positive African sera tested by ELISA IgG, all but one were positive by ELISA IgG. Sixty-four African sera negative by PRNT were also negative by ELISA IgG. There was a significant correlation ($r = 0.74$, $P < 0.01$) between PRNT titer and ELISA IgG OD reading (Fig. 2) for both groups.

A total of 57 sera from Bedouin living in the Sinai were tested by PRNT, HI, and ELISA IgG. All 57 were negative by PRNT, 28 (49%) had an HI titer of 1:10, and 22 (39%) had an HI titer of 1:20, a result considered as nonspecific inhibition of hemagglutination. All 57 were negative by ELISA IgG. Nine sera with positive titers to Sandfly fever Sicilian virus (PRNT titers of 1:10 to 1:160) were all negative by ELISA IgG.

ELISA for specific IgM compared with PRNT. A total of 76 sera from 21 individuals immunized with RVF vaccine were tested by PRNT and ELISA μ-capture IgM technique. Sera from day 0 ($n = 21$) were all negative. Sera from days 6 to 8 ($n = 23$) were negative by PRNT and negative or weakly positive by ELISA IgM. Sera from days 32 to 34 ($n = 19$) and days 42 to 52 ($n = 13$) were positive by both PRNT and ELISA IgM. Figure 3 shows the development of RVFV antibodies by time as measured with ELISA μ-capture IgM, ELISA IgG, and PRNT. Sera collected at 6 months postvaccination ($n = 15$) and African sera ($n = 26$), all positive by PRNT, were negative by ELISA IgM.

Of 37 postvaccination sera, 6 were strongly positive, 10 were weakly or border-line positive, and 21 were negative by ELISA μ-capture IgM; they were also tested by the indirect ELISA, using anti-human IgM conjugate. Both ELISA IgM
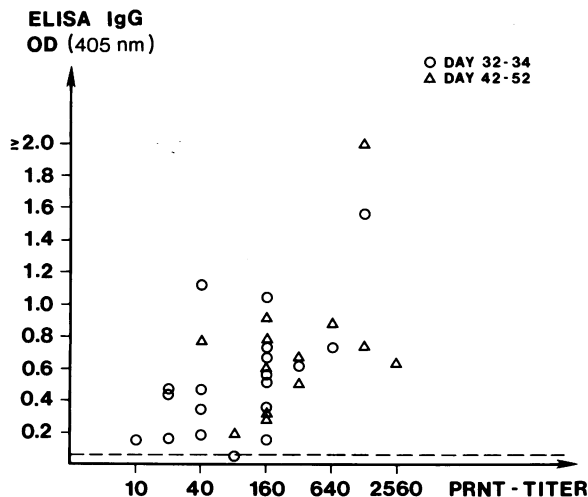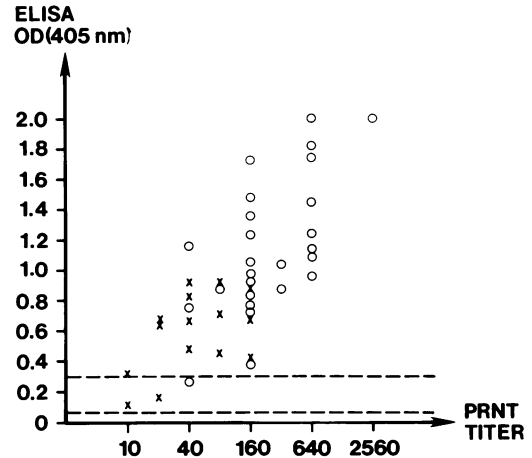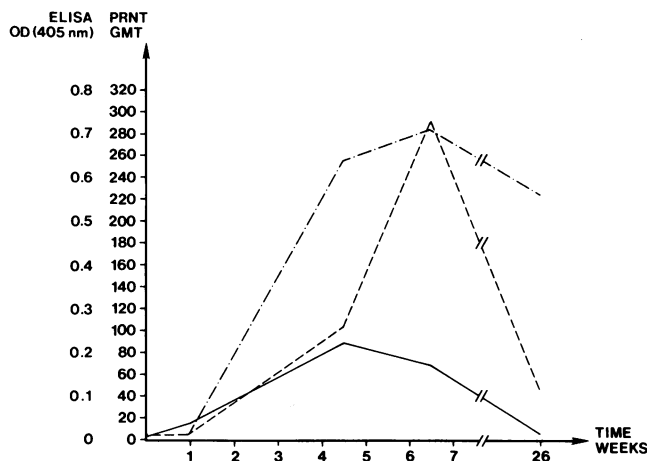

FIG. 1. Comparison of ELISA and PRNT in early postvaccination sera. ELISA IgG OD reading compared with PRNT titer of sera collected on days 32 to 34 and 42 to 52 after RVF vaccination.

FIG. 3. Development of RVF antibodies after vaccination. Sera were tested on days 0, 6 to 8, 30 to 34, and 40 to 51 by ELISA μ-capture IgM (mean OD), ELISA IgG (mean OD), and PRNT. Symbols: ——, ELISA IgM; —·—·—·, ELISA IgG; ·······, PRNT geometric mean titer.

techniques gave almost the same OD readings with similar antibody patterns, with sera from days 32 to 34 giving higher values than those from days 42 to 52.

Protein A-adsorbed sera from days 6 to 8, 32 to 34, and 42 to 52 had lower OD readings compared with untreated sera but otherwise showed exactly the same pattern; days 6 to 8 were negative or weakly positive and days 32 to 34 were positive with a higher OD reading than that on days 42 to 52.

DISCUSSION

The recent RVF epidemic in Egypt demonstrated the potential for the disease to extend its geographic boundaries. Rapid diagnostic tools are needed to detect RVF in both humans and animals in known endemic regions and potentially receptive extension zones (17). Travelers from Africa may introduce the disease to other parts of the world (13). A double sandwich ELISA for RVFV antigen detection has been reported (12). However, an antibody assay is also needed for detection of specimens collected from patients after the viremic phase, particularly since retinal vasculitis and encephalitis occur after the initial illness. These assays could also be applied to epidemiological surveillance and measurement of antibody response after vaccination.

Several methods are available for the detection of RVF antibodies, e.g., the HI, CF, indirect immunofluorescence, and agar gel diffusion tests and PRNT. These methods are all useful for measuring antibodies soon after an RVF infection. The HI test has given poor results with vaccinees in some reports (14) but has been satisfactory when studied in other laboratories (9, 22; C. J. Peters, J. M. Meegan, and G. J. Urbanski, unpublished data), although it is consistently less sensitive than PRNT (7, 14). Our results confirm these findings. The neutralization test, although sensitive and specific (16, 21), requires cell culture or animal facilities. Furthermore, work with live RVFV outside endemic regions requires special containment laboratories (15).

ELISA for RVFV IgG proved to be almost as sensitive as PRNT when used on postvaccination sera. It was more sensitive than HI or CF, which failed to detect antibodies in most specimens positive in PRNT and ELISA IgG. There were no false positive results in the ELISA IgG test.

High-background activity due to nonspecific binding is a well-known problem in most ELISA systems. A high frequency of so-called "sticky sera" has been found during seroepidemiological studies in Africa. Sera collected in Sierra Leone and the Central African Republic gave a much higher OD reading in the control (test without antigen) compared with sera from Swedish soldiers. However, false-positive readings due to nonspecific binding are minimized by subtracting the control OD reading from the test result. The greater variation in background activity in African sera is important to note since the usual procedure used (mean plus standard deviations) to estimate the cut-off between positive and negative results will cause it to increase considerably. Figure 3 shows correlation between ELISA IgG OD readings and PRNT titers for both postvaccination antibodies and antibodies after a natural infection.

There are serological cross-reactions between RVF and other viruses in the Sandfly fever (phlebovirus) virus group when studied with the HI and IF tests (16). A limited number of convalescent sera with antibodies to one member of the phleboviruses, Sandfly fever (Sicilian), were tested for cross-reaction in ELISA IgG. As an additional measure of specificity, 22 sera with borderline RVF HI titers of 1:20 but no neutralizing antibodies were selected. All of these specimens were negative by the ELISA IgG.

In the acute phase of an infection, or shortly thereafter, the detection of specific IgM antibodies would be a useful diagnostic tool. We found that all sera collected 1 month after initiation of an RVF vaccination program were positive by both ELISA IgM tests employed. Sera collected 6 to 8 weeks postvaccination had, in general, a lower ELISA IgM OD reading than the sera collected at 4 weeks.

It would be anticipated that ELISA μ-capture IgM is superior to a system with specific anti-IgM conjugates since it avoids the problem with interacting specific IgG. However, to determine whether a high concentration of specific IgG could interfere with IgM detection, one specimen with a high ELISA IgM reading (day 32) was diluted 1:40 in ELISA buffer containing 10% human RVF convalescent serum (PRNT titer of 1:2,560). The serum became negative in the indirect ELISA IgM test but gave the same OD as the original sample when ELISA μ-capture IgM was used (data not shown). Our comparison of the two ELISA IgM techniques showed little difference when postvaccination sera were used.

The presence of rheumatoid factor in serum may also cause false-positive results in the IgM test. Protein A can be used to adsorb IgG from sera before testing, avoiding false-positive results due to the rheumatoid factor. This treatment decreased reactivity in the RVFV IgM test, presumably because protein A also adsorbs parts of the IgM (19).

The two ELISA methods (IgG and μ-capture IgM) merit consideration for field evaluation since they provide a rapid, specific, and sensitive test to detect antibodies. More study is needed with field-collected sera, cross-reactions with other phleboviruses, kinetics of antibody formation after infection, and use in nonhuman species. The ELISA test may not be superior to the neutralization test but it can be standardized and performed entirely with inactivated reagents, increasing its utility in surveillance in nonendemic areas.

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